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Post-transplant hypophosphatemia: Tertiary 'Hyper-Phosphatoninism'?

I Bhan[1], A Shah[1], J Holmes[1], T Isakova[2], O Gutierrez[2], S-A Burnett[2], H Jüppner[2,3] and M Wolf[1]

[1]Renal Unit, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA; [2]Endocrine Unit, Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA and [3]Pediatric Nephrology Unit, Departments of Medicine and Pediatrics, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA

Hypophosphatemia is a common complication of kidney transplantation. Tertiary hyperparathyroidism has long been thought to be the etiology, but hypophosphatemia can occur despite low parathyroid hormone (PTH) levels and can persist after high PTH levels normalize. Furthermore, even in the setting of normal allograft function, hypophosphatemia, and hyperparathyroidism, calcitriol levels remain inappropriately low following transplantation, suggesting that mechanisms other than PTH contribute. Fibroblast growth factor-23 (FGF-23) induces phosphaturia, inhibits calcitriol synthesis, and accumulates in chronic kidney disease. We performed a prospective, longitudinal study of 27 living donor transplant recipients to test the hypotheses that excessive FGF-23 accounts for hypophosphatemia and decreased calcitriol levels following kidney transplantation. Hypophosphatemia <2.5 mg/dl developed in 85% of subjects, including one who had previously undergone parathyroidectomy; 37% developed phosphate $\leqslant 1.5$ mg/dl. The mean pre-transplant FGF-23 level was $1,218 \pm 542$ RU/ml. Within the first week following transplantation, mean levels decreased to 557 ± 579 RU/ml, which were still above normal. FGF-23 was independently associated with serum phosphate ($P<0.01$), urinary excretion of phosphate ($P<0.01$), and calcitriol levels ($P<0.01$); PTH was not independently associated with any of these parameters. We calculated area under the curve for FGF-23 and PTH between the pre- and first post-transplant levels as a summary measure of early exposure to these phosphaturic hormones. An area under the FGF-23 curve greater than the median was associated with a relative risk of developing hypophosphatemia $\leqslant 1.5$ mg/dl of 5.3 ($P=0.02$) compared with lower levels. Increased area under the PTH curve was not associated with greater risk of hypophosphatemia. Excessive FGF-23 exposure in the early post-transplant period appears to be more strongly associated with post-transplant hypophosphatemia than PTH.

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Correspondence: *M Wolf, Renal Unit, Massachusetts General Hospital, Harvard Medical School, Bartlett 917, 55 Fruit Street, Boston, Massachusetts 02114, USA. E-mail: mswolf@partners.org*

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Hypophosphatemia owing to inappropriate urinary phosphate wasting is a common complication of kidney transplantation, occurring in up to 93% of patients during the first few months after transplantation.[1,2] Although this phenomenon is usually limited to the early post-transplant period, hypophosphatemia may persist for more than 10 years.[3] Persistently increased levels of parathyroid hormone (PTH) due to secondary hyperparathyroidism associated with advanced chronic kidney disease (CKD) have long been thought to be the etiology of post-transplant hypophosphatemia, that is, tertiary hyperparathyroidism.[2] However, hyperparathyroidism does not appear to be the only mechanism as inappropriate urinary phosphate wasting can occur despite low levels of PTH, and hypophosphatemia can persist after elevated PTH levels have normalized.[4–7] Furthermore, calcitriol levels are often inappropriately low following renal transplantation despite normal allograft function, hypophosphatemia, and hyperparathyroidism, which should stimulate increased calcitriol synthesis.[8–10] Therefore, mechanisms other than PTH likely contribute to the syndrome of hypophosphatemia, urinary phosphate wasting, and inappropriately low calcitriol levels following kidney transplantation.

Fibroblast growth factor-23 (FGF-23) induces phosphaturia and inhibits renal 1-α hydroxylase leading to decreased calcitriol synthesis.[11] Disorders of FGF-23 excess are characterized by hypophosphatemia with inappropriate renal phosphate wasting and inappropriately low calcitriol levels for the degree of hypophosphatemia. Examples include transgenic mice engineered to overexpress FGF-23, mice administered exogenous FGF-23, and humans with autosomal-dominant hypophosphatemic rickets, fibrous dysplasia, tumor-induced osteomalacia, and many cases of X-linked hypophosphatemia.[11–18] Recent studies suggest that FGF-23 levels increase as CKD progresses and contribute to the declining calcitriol levels observed in CKD.[19–21] We hypothesized that persistently increased FGF-23 levels following kidney transplantation contribute to the syndrome of post-transplant hypophosphatemia by increasing tubular phosphate wasting and suppressing calcitriol synthesis by inhibiting renal 1-α

hydroxylase. We compared the strength of association between these variables and FGF-23 relative to PTH.

RESULTS

Characteristics of the study population

The characteristics of the study population are presented in Table 1. The immune suppression prescription was determined according to the Massachusetts General Hospital (MGH) Transplant Unit's departmental protocols. Most subjects were treated with thymoglobulin induction, beginning intraoperatively and for a total of three doses within the first 3 days. All subjects except one were prescribed corticosteroids, mycophenolate mofetil, and tacrolimus for maintenance of immune suppression. These agents were initiated immediately before the transplant and were dosed according to an identical internal dosing protocol. One subject did not receive tacrolimus or mycophenolate but instead underwent a combined bone marrow kidney transplant as part of an experimental immune tolerance protocol.

Phosphorus and vitamin D metabolism post-transplantation

Baseline, pre-transplant laboratory test results are presented in Table 2. Figure 1 illustrates the scatter plots for serum creatinine (a), potassium (b), phosphate (c), fractional excretion of phosphate ($FEPO_4$) (d), FGF-23 (e), PTH (f), and calcitriol (g) plotted against the days post-transplant. Pre-transplant levels are plotted at time = 0. Serum creatinine levels normalized quickly in the majority of subjects by the time of their first post-transplant blood sampling (Figure 1a). There were only five episodes of hypokalemia (serum potassium was <3.4 meq/l) during the study period (Figure 1b). In one subject this was present pre-transplant, and another subject accounted for two of the remaining four episodes of hypokalemia. All instances of post-transplant hypokalemia occurred after day 27, long after the acute reduction in serum creatinine that accompanies the osmotic diuresis immediately following transplantation. In contrast, 85% of subjects developed post-transplant hypophosphatemia (Figure 1c), defined as a serum phosphate <2.6 mg/dl; 37% developed severe post-transplant hypophosphatemia, defined as a level $\leqslant 1.5$ mg/dl. The dissociation between hypophosphatemia and hypokalemia suggests that post-transplant hypophosphatemia was not caused by nonspecific tubular dysfunction.

The mean pre-transplant FGF-23 level was 1218 ± 542 RU/ml. Mean FGF-23 levels decreased to 557 ± 579 RU/ml within the first week following transplantation (Figure 1e), but these levels remained significantly above mean levels reported in healthy populations (55 ± 50–59 ± 42 RU/ml).[16,22] After transplant, no subjects developed hypercalcemia and none were treated with sirolimus, cyclosporine, phosphorus supplements, phosphorus binders, nutritional vitamin D supplements, active vitamin D preparations, or cinacalcet.

The results of the regression analyses are presented in Table 3. Both FGF-23 and PTH were associated with the serum phosphate in univariate analyses. When both were included together in a multivariate model, only FGF-23 remained independently associated with serum phosphate levels. Only FGF-23 was independently associated with $FEPO_4$ in both univariate and multivariate analyses. Vitamin D deficiency, defined as a 25D level <30 ng/ml, was present in 20 of the 27 subjects at the time of transplantation but only one subject was severely deficient ($\leqslant 10$ ng/ml) with a level of 9.7 ng/ml. Although 25D deficiency is a known cause of secondary hyperparathyroidism,[23] there was no association between 25D levels and PTH ($r = -0.03$; $P = 0.9$), and baseline 25D levels were not associated with increased risk of developing hypophosphatemia. FGF-23 was inversely related

Table 1 | Patient characteristics (*n*=27)

Age (mean ± s.d.)	**44.5 ± 11.8**
Gender (n)	
Women	12
Men	15
Race (n)	
Caucasian	23
African-American	3
Hispanic	1
Etiology of kidney disease (n)	
Diabetes mellitus	3
Hypertension	2
Glomerulonephritis	11
Polycystic kidney disease	4
Other	7
Transplant (n)	
First	19
Second	8
Donor (n)	
Living related	15
Living unrelated	12
Previous treatment (n)	
Dialysis	18
Pre-emptive transplant	9
Sensitization (n)	
None	9
0–50%	16
>50%	2
Induction therapy (n)	
None	6
Thymoglobulin	21

s.d., standard deviation.
Continuous variables are expressed as mean ± s.d.

Table 2 | Results of baseline, pre-transplant laboratory tests

Creatinine (mg/dl)	6.8 ± 2.3
Phosphate (mg/dl)	5.3 ± 1.7
Intact parathyroid hormone (pg/ml)	305 (123, 546)
FGF-23 (RU/ml)	1218 ± 542
25(OH) vitamin D3 (ng/ml)	23 ± 8
1α,25(OH)$_2$ vitamin D3 (pg/ml)	13.5 (8, 27)

FGF, fibroblast growth factor.
Normally distributed variables are expressed as mean ± s.d., others as median (25th and 75th percentiles).

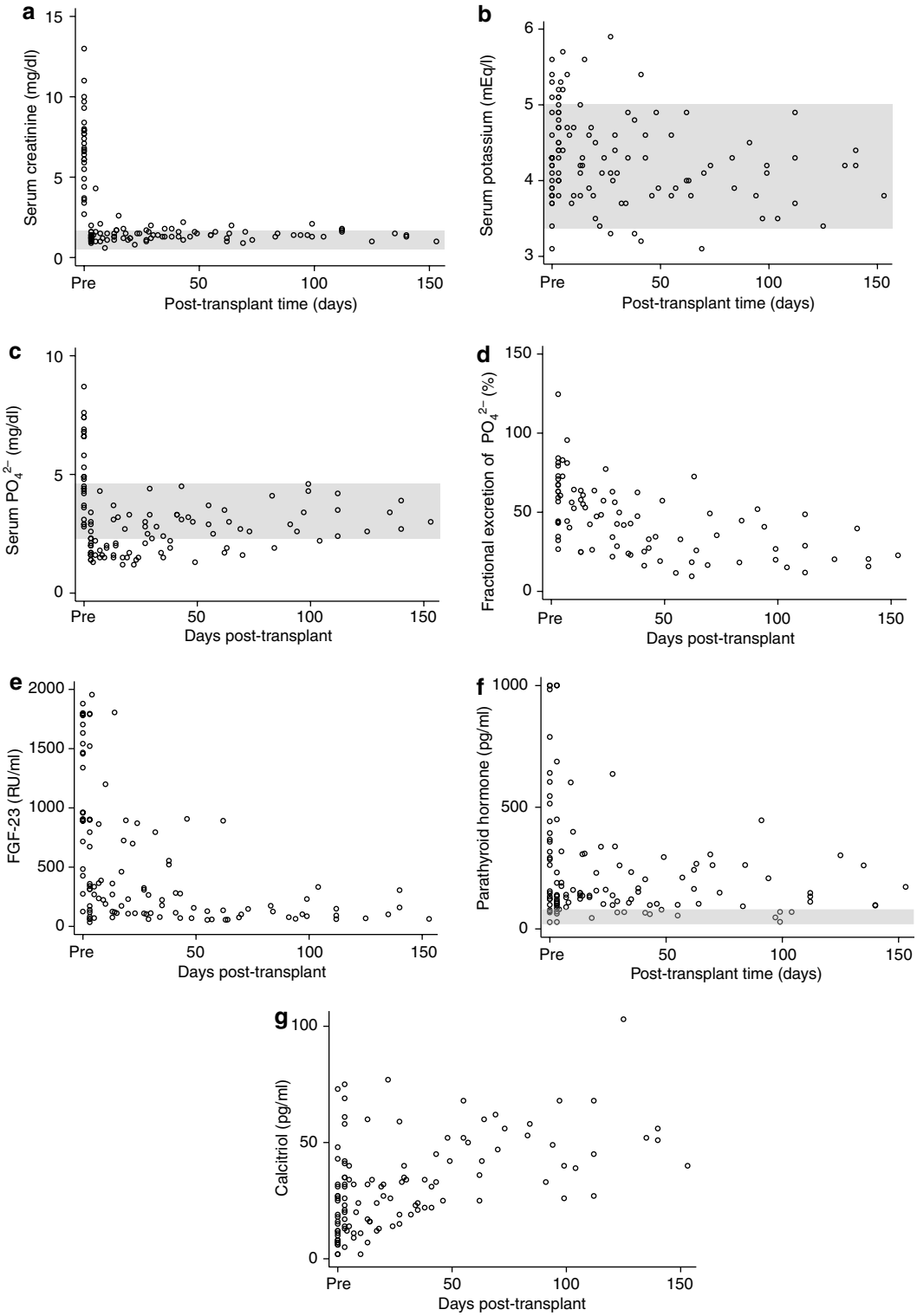

Figure 1 | Scatter plots for (a) serum creatinine, (b) potassium, (c) serum phosphate, (d) $FEPO_4$, (e) FGF-23, (f) PTH, and (g) calcitriol plotted against the days post-transplant. Pre-transplant levels are plotted at time = 0. The shaded area in (**b**, **c**, and **f**) represents the normal range of these variables.

to calcitriol and was the only independent predictor of calcitriol levels in univariate analyses; there was no association between calcitriol and PTH or 25D levels (Table 3). In a multivariate model adjusting for 25D levels, FGF-23 remained the only parameter that was independently associated with calcitriol (inversely). To further exclude the possibility that 25D deficiency affected the results, we repeated the regression analyses after excluding subjects with

Table 3 | Results of the regression analyses modeling serum phosphate, fractional excretion of phosphate, and calcitriol levels

	Univariate				Multivariate			
	β	s.e.	*z*	*P*-value	*β*	s.e.	*z*	*P*-value
Dependent variable: Serum phosphate levels								
FGF-23	0.001	<0.001	3.93	<0.001	0.001	<0.001	3.84	<0.001
PTH	0.001	<0.001	1.97	0.05	0.001	<0.001	1.51	0.13
Dependent variable: Fractional excretion of phosphate								
FGF-23	0.011	0.005	2.44	0.01	0.011	0.005	2.38	0.02
PTH	0.015	0.012	1.29	0.2	0.019	0.011	1.68	0.09
Dependent variable: Calcitriol levels								
FGF-23	−0.11	0.03	−4.00	<0.001	−0.11	0.03	−3.84	<0.001
$FGF\text{-}23^2$	<0.001	<0.001	3.06	0.002	<0.001	<0.001	2.82	0.005
$FGF\text{-}23^3$	<−0.001	<0.001	−2.54	0.01	<−0.001	<0.001	−2.27	0.02
PTH	0.005	0.007	0.68	0.5	0.009	0.007	1.31	0.2
25D	0.04	0.26	0.15	0.9	0.20	0.23	0.86	0.4

β, beta coefficient; 25D, $25(OH)_2$ vitamin D; FGF, fibroblast growth factor; PTH, parathyroid hormone; s.e., standard error; z, z statistic.
All models account for individual subjects as a random effect and days post-transplant as the repeated-measures time factor. *β* coefficients refer to change in dependent variable per unit increase in the independent variables: per RU/ml for FGF-23; per pg/ml for PTH; per ng/ml for 25D.

25D levels <15 ng/ml. This left 22 subjects in the analysis but did not change the results. FGF-23 alone remained independently associated with serum and urinary phosphate levels and calcitriol levels during the post-transplant period.

FGF-23 versus PTH in post-transplant hypophosphatemia

Five subjects had PTH levels <100 pg/ml before transplant. Nonetheless, four of these subjects developed hypophosphatemia with phosphate levels <2.0 mg/dl; two developed severe hypophosphatemia (⩽1.5 mg/dl). Interestingly, one of these subjects had undergone a subtotal parathyroidectomy for severe secondary hyperparathyroidism before transplantation. His pre-transplant PTH was 133 pg/ml and FGF-23 was 1800 RU/ml. On post-transplant day 4, he developed hypophosphatemia with a serum phosphate of 2 mg/dl. The subject who never received tacrolimus also developed hypophosphatemia with a nadir of 1.4 mg/dl.

To further evaluate the relative importance of FGF-23 versus PTH in the development of severe post-transplant hypophosphatemia, we examined the likelihood of developing a serum phosphate level ⩽1.5 mg/dl according to three different measures of FGF-23 and PTH exposure: the absolute pre-transplant levels, the immediate percent reduction in levels post-transplant, and the overall hormone exposure during the early post-transplant period measured by the area under the FGF-23 and PTH curves. There was a nonsignificant trend towards increased risk of developing severe post-transplant hypophosphatemia among subjects whose pre-transplant FGF-23 levels were above the median compared to those below the median (relative risk (RR) = 2.5; $P = 0.08$). Subjects whose pre-transplant PTH levels were above the median were not at increased risk of developing severe post-transplant hypophosphatemia compared to those with levels below the median (RR = 0.43; $P = 0.10$).

Next, we examined whether severe post-transplant hypophosphatemia might be linked to persistent elevations of FGF-23 or PTH rather than the magnitude of elevation in the baseline, pre-transplant levels (Figure 2). First, we analyzed the risk of severe post-transplant hypophosphatemia according to the percent change in individual subjects' FGF-23 and PTH from the pre- to the first post-transplant levels. Compared to those subjects whose initial reduction in FGF-23 levels was above the median, those whose FGF-23 levels persisted at higher levels (percent reduction in FGF-23 levels below the median) were more likely to develop hypophosphatemia (RR = 3.2; $P = 0.05$). In contrast, there was no association between the magnitude of the initial reduction in PTH levels and subsequent risk of severe hypophosphatemia: subjects with persistent elevation of PTH (percent decline in PTH levels below the median) had a RR of developing severe hypophosphatemia of 1.3 ($P = 0.7$) compared to those with a percent decline in PTH levels above the median.

Finally, we examined area under the FGF-23 and PTH curves spanning the pre- and first post-transplant levels as a summary measure of subjects' overall initial exposure to these hormones. The RR of developing severe hypophosphatemia was 5.3 ($P = 0.02$) among those whose area under the FGF-23 curve was greater than the median compared to those below the median. In contrast, the RR of developing severe hypophosphatemia was less likely (RR = 0.2; $P = 0.01$) among those whose area under the PTH curve was greater than the median compared to those below the median, the opposite of what would be expected if post-transplant hypophosphatemia was mediated by excessive PTH exposure.

DISCUSSION

In this prospective longitudinal study of 27 patients undergoing living donor kidney transplantation, FGF-23 rather than PTH appeared to be the primary factor accounting

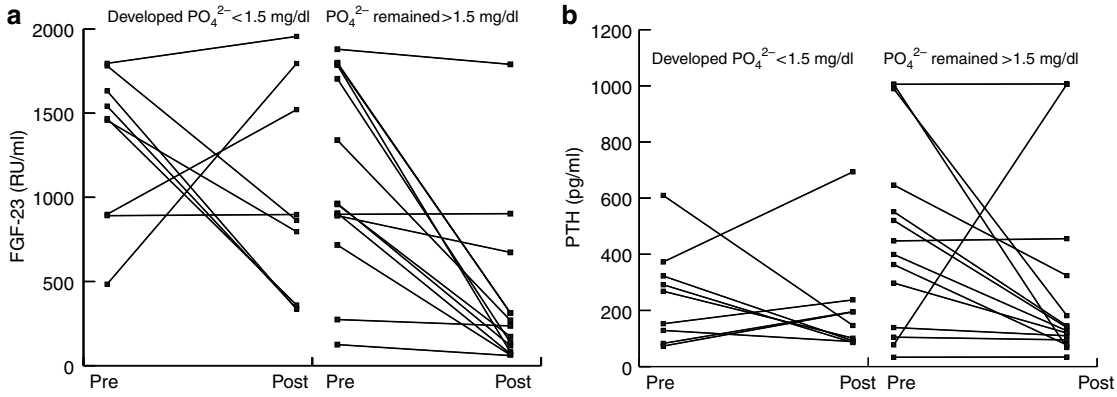

Figure 2 | Pre- and first post-transplant (a) FGF-23 and (b) PTH levels in subjects who did or did not develop severe hypophosphatemia ($\leqslant$1.5 mg/dl). Individual subjects' levels are connected. (**a**) The RR of developing severe hypophosphatemia was 5.3 ($P = 0.02$) among those whose area under the FGF-23 curve was greater than the median compared to those below the median. (**b**) The RR of developing severe hypophosphatemia was 0.2 ($P = 0.01$) among those whose area under the PTH curve was greater than the median compared to those below the median.

for the syndrome of post-transplant hypophosphatemia. Increased FGF-23 but not PTH levels were independently associated with decreased serum phosphate levels and increased phosphate wasting marked by increased $FEPO_4$. The risk of developing severe hypophosphatemia was more tightly linked to subjects' overall exposure to FGF-23 during the immediate post-transplant period compared with the corresponding values for PTH. Furthermore, hypophosphatemia developed in four of the five subjects with low PTH levels before transplantation including one who had undergone a subtotal parathyroidectomy. In addition to these results, a critical observation was the strong inverse association between FGF-23 excess and persistently decreased calcitriol levels. Although increased PTH levels could confound the associations between FGF-23 and serum and urinary phosphate levels, low calcitriol levels persisted following transplantation, despite excessive PTH, a healthy allograft, and hypophosphatemia, each of which should have stimulated calcitriol production. This finding supports an independent inhibitory effect of FGF-23 on renal 1-α hydroxylase. Collectively, these results support a leading role for FGF-23 in the development of hypophosphatemia and decreased calcitriol levels following kidney transplantation, and add to the growing body of evidence that implicate FGF-23 in a variety of human disorders of phosphate metabolism.

To date, FGF-23 has been implicated in the pathogenesis of several rare disorders of abnormal phosphate homeostasis that are characterized by phosphate wasting, hypophosphatemia, and relative calcitriol deficiency and that were initially thought to be due to elusive 'phosphatonins.' For example, most patients with tumor-induced osteomalacia have mesenchymal tumors that secrete excessive FGF-23.[12] Likewise, hypophosphatemia in patients with fibrous dysplasia is due to excessive secretion of FGF-23 by dysplastic bone lesions.[15] In autosomal-dominant hypophosphatemic rickets, a mutation in the FGF-23 cleavage site renders it resistant to inactivation leading to excessive accumulation of the hormone.[24,25] FGF-23 levels are also increased in most patients with X-linked hypophosphatemia, although the pathogenic link between mutations in the PHEX gene (phosphate-regulating gene with homologies to endopeptidases on the X chromosome) and excess FGF-23 remains incompletely understood.[16]

Based on these results in rare diseases, subsequent studies demonstrated a role for FGF-23 in normal phosphorus metabolism[22,26] and in the pathogenesis of nephrolithiasis.[27] Furthermore, FGF-23 levels increase as kidney function declines and are independently associated with early and progressive calcitriol deficiency[21] and thus may contribute to the pathogenesis of secondary hyperparathyroidism in CKD. Dialysis patients, whose chronic hyperphosphatemia is thought to stimulate constitutive FGF-23 secretion, demonstrate the most dramatically increased FGF-23 levels.[19,28] Based on the current study, we propose that post-transplant hypophosphatemia is attributable in part to the persistence of this excessive FGF-23 secretion into the post-transplant period, resulting in urinary phosphate wasting by the healthy allograft that, unlike the native kidney, is capable of responding to the phosphaturic hormone. This phenomenon is analogous to post-transplant hypercalcemia caused by persistent or tertiary hyperparathyroidism. It is still unclear why excessive FGF-23 secretion persists post-transplant, how involution of excessive FGF-23 secretion eventually occurs and why, in some cases, hypophosphatemia can persist for months following transplantation.[3] In adults, FGF-23 is produced mostly by bone.[29] Perhaps uremic bone develops resistance to the inhibitory stimuli to decrease FGF-23 secretion because of the preceding months to years of excessive FGF-23 stimulation.

FGF-23 suppresses sodium-dependent phosphate reabsorption by inhibiting the expression of the types IIa and IIc sodium-dependent phosphate co-transporters in the brush border of renal proximal tubules.[30] PTH induces phosphaturia through a similar mechanism, but studies

of parathyroidectomized animals demonstrate that the phosphaturic effect of FGF-23 is independent of PTH.[31,32] Although our data support an independent effect of FGF-23 on renal phosphate handling, differentiating the effects of FGF-23 from those of PTH in human studies is challenging, especially when levels of both hormones are elevated as in the post kidney transplant model. We had hoped to recruit additional subjects who had previously undergone parathyroidectomy, but only one such additional patient underwent a transplant during the recruitment period and she declined participation. Nevertheless, studying calcitriol levels following kidney transplantation provides an opportunity to isolate the effects of FGF-23 from PTH *in vivo* because PTH stimulates the renal 1α-hydroxylase whereas FGF-23 is a direct inhibitor.[32,33] The observation that calcitriol levels remain low in association with increased FGF-23 despite normal allograft function and the stimulatory effects of hypophosphatemia and hyperparathyroidism highlights the central regulatory role of FGF-23. This observation is supported by the finding that mice which are genetically engineered to express excessive FGF-23 demonstrate persistent inhibition of 1α-hydroxylase activity, despite the development of secondary hyperparathyroidism.[34] Given the pleiotropic effects of calcitriol, including its effects on the immune system that may beneficially impact allograft survival,[35,36] further studies are needed to explore novel methods of interrupting those factors which inhibit renal 1α-hydroxylase and thereby delay the recovery of normal calcitriol levels.

A limitation of the study is that we measured FGF-23 levels using an assay that detects C-terminal fragments along with the biologically active molecule instead of using the intact FGF-23 assay that has recently become available. Although C-terminal fragments may accumulate preferentially as renal function declines, for the same reason, they are likely to be eliminated more quickly following transplantation for the same reason. Importantly, the longitudinal, repeated-measures design limited to subjects who experienced prompt allograft function enabled us to observe significant associations between FGF-23 and the outcome variables, despite potential imprecision in the assay which would increase variability. In addition, FGF-23 is one of the several putative phosphatonins that could contribute to post-transplant hypophosphatemia. Additional studies are needed to measure the effects of other phosphatonins such as matrix extracellular phosphoglycoprotein, frizzled related protein 4, and FGF-7.[29,37]

Likewise, the PTH assay we used detects both the 'bio-intact' 1–84 molecule and 7–84 fragments. Although certain reports suggest that PTH 7–84 may antagonize the actions of PTH 1–84 on bone,[38] other investigators were unable to demonstrate a clear effect of 7–84 levels on bone histology.[39,40] There is even less data on the effects of 7–84 fragments on phosphate transport and calcitriol metabolism. Accordingly, the therapeutic targets for PTH levels recommended by international management guidelines are based on the intact PTH assay we used,[41,42] which remains the standard assay for clinical trials of secondary hyperparathyroidism in CKD.[43,44] Furthermore, prior data in patients with renal failure have demonstrated nearly complete correlation ($r = 0.92$) between the intact PTH assay we used and the 'bio-intact' PTH assay that detects only PTH 1–84.[43] Although the absolute concentrations of PTH would have differed using a 'bio-intact' assay, the results would have been collinear with the results we obtained and thus, would have been unlikely to alter the associations we observed.

Confounding by the immunosuppressive drugs that subjects received is another potential limitation. Although high-dose steroids and tacrolimus have been linked to renal phosphate wasting,[7,45,46] hypophosphatemia is common following kidney transplantation but does not typically occur after other solid organ transplants where similar immunosuppressive regimens are used, often in higher doses.[47] This suggests that although immunosuppressive agents might exacerbate phosphaturia following kidney transplantation, they are unlikely to be the primary pathogenic factor. In the current study, several factors argue against significant confounding by the immune suppression. Nearly all patients were on an identical immunosuppressive regimen, yet not all patients developed hypophosphatemia. In those affected, the severity varied despite the homogeneity of the treatment protocol. During the course of the longitudinal follow-up, hypophosphatemia resolved yet the immunosuppressive agents that were administered remained. One subject who never received tacrolimus or mycophenolate developed hypophosphatemia. Furthermore, the significant associations we observed between several indices of phosphorus homeostasis and FGF-23 would not be expected if the phosphate losses were nonspecifically related to extraneous factors such as immune suppression. Finally, although immunosuppressive drugs have been linked to phosphate wasting in some studies, there are limited data to suggest that these agents confound the strong association we observed between increased FGF-23 and decreased calcitriol levels.

Vitamin D deficiency (25D < 30 ng/ml) is common in the general population, in patients with kidney disease, and was also common in our study population.[23,48] We do not believe, however, that low levels of 25D significantly affected the results. Although 25D levels of 15–30 ng/ml are associated with modestly increased PTH levels,[23] we are unaware of any reports that indicate an increased risk of hypophosphatemia in patients with a mild reduction in 25D levels. Furthermore, hypophosphatemia in patients with severe vitamin D deficiency is mediated by PTH-induced urinary phosphate wasting in addition to decreased intestinal absorption of dietary phosphorus. In this study, there was no correlation between PTH and 25D levels, and we found no association between PTH levels and phosphaturia or hypophosphatemia. Similarly, there was no association between baseline 25D levels and subsequent risk of developing mild or severe hypophosphatemia. We adjusted the multivariate models for 25D levels and this did not alter the results. Similarly, the

results did not change when the analyses were restricted to subjects with 25D levels >15 ng/ml. Decreased 25D levels appear to have had minimal impact on our results.

It could be argued that post-transplant hypophosphatemia represents more of a laboratory test nuisance rather than a clinically important complication of kidney transplantation. The primary goals of this study, however, were to examine the hormonal mechanisms underlying post-transplant hypophosphatemia and to obtain novel insight into the *in vivo* biology of FGF-23 in humans. We do not propose FGF-23 as a diagnostic tool to predict hypophosphatemia in transplant recipients, and we did not aim to study the incidence of hypophosphatemia after living versus cadaveric donor transplants. Furthermore, although post-transplant hypophosphatemia is usually self-limited and asymptomatic in most cases, there are reports of patients with protracted, symptomatic hypophosphatemia that is difficult to treat.[3] Designing appropriate treatment strategies for those patients requires further understanding of disease mechanisms. The current study might explain the shortcomings of our current management strategies. High doses of phosphorus supplements and active vitamin D preparations both induce increased FGF-23 secretion and thus promote further phosphaturia, which would seem to offset their benefit.[49] Unfortunately, the current study does not suggest new management approaches that can be immediately integrated into clinical practice. The development of novel approaches for the future will require further insight into the regulation of FGF-23 and its receptor in order to learn how to facilitate more rapid involution of its secretion or mechanisms of blocking its effects on the kidney.

MATERIALS AND METHODS

Study population

We performed a longitudinal prospective cohort study of 27 patients undergoing living donor kidney transplantation at MGH. The Transplant Unit at MGH serves a diverse patient population from Boston and surrounding areas. All patients aged 18 years or older were eligible for the study. Subjects were excluded if they had a hematocrit <27%, a preexisting phosphate wasting disorder, or if they underwent cadaveric transplantation. Patients undergoing cadaveric transplantation were excluded because of the greater risk of delayed graft function with associated oliguria and hyperphosphatemia that would confound the analyses. In addition, the uniform pre-transplant schedule in living recipients allowed written informed consent to be obtained at the last pre-transplant visit 1 week before the procedure. The study adhered to the Declaration of Helsinki Principles and was approved by the Human Research Committee at MGH.

Procedures, assays, and calculations

Blood and urine samples were collected before transplantation and longitudinally thereafter. The first post-transplant specimens were collected at the time of the creatinine nadir, which was typically 4–5 days post-transplant. Subsequently, samples were collected every 2 weeks during the first 2 months, then at 3 and 6 months post-transplant coinciding with routine follow-up visits to the Transplant Clinic. To minimize confounding, sample collection on individual patients was discontinued if they developed allograft dysfunction. Three subjects developed acute rejection necessitating discontinuation of blood sampling. Blood sampling was discontinued prematurely in another subject who developed significant anemia. Six subjects from remote New England areas resumed their care with their referring nephrologists near their homes after completing 3 months of care at the transplant center. Thereafter, these six subjects were unavailable to continue their sample collections for the study. A total of 119 observations were collected in the 27 subjects; the mean number of follow-up visits was 4. 4 per subject and the follow-up ranged from 4 to 153 days post-transplant.

After collection, samples were stored for less than 2 h at 5°C. Upon arrival to the laboratory, the plasma and serum samples were centrifuged at 3200 r.p.m. in a Beckman Coulter Spintrol DLX centrifuge for 9 min, aliquoted, and stored at −80°C for future analysis. Urine samples were shaken, aliquoted, and also stored at −80°C. When enrollment was complete, the stored samples underwent a single thaw followed by batched assay of individual biomarkers in the Core Laboratory of the Mallinckrodt General Clinical Research Center at MGH.

FGF-23 was measured using a two-site enzyme-linked immunosorbent assay that uses affinity purified anti-FGF-23 antibodies directed against two epitopes within the carboxy-terminal portion of FGF-23 (Immutopics, San Clemente, CA, USA); this assay measures intact FGF-23 as well as C-terminal fragments.[16] The inter- and intra-assay coefficients of variation were 7.3 and 5.0%, respectively. The assay has been used in previous studies of CKD patients.[19,21] PTH was measured using a two-site, second-generation enzyme-linked immunosorbent assay that detects intact PTH (Immutopics, San Clemente, CA, USA), with inter- and intra-assay coefficients of variation of 7.7 and 3.2%, respectively.[39] Calcitriol was measured using a radioimmunoassay (DiaSorin Inc., Stillwater, MN, USA) with coefficients of variation <6.5% at concentrations <32.5 pg/l. In order to adjust for differences in vitamin D stores, we also measured baseline serum $25(OH)_2$ vitamin D3 (25D) levels using a radioimmunoassay (DiaSorin Inc., Stillwater, MN, USA). The coefficient of variation for the 25D assay was <2.3% at a range of <30 ng/ml. Serum creatinine, calcium, phosphate and urine creatinine, and phosphate were measured using routine assays. The $FEPO_4$ was the primary measure of renal phosphate handling.[50] $FEPO_4$ is calculated by dividing the ratio of urine to serum phosphate by the ratio of urine to serum creatinine. This calculation standardizes urinary phosphate excretion for differences in urine concentration and the simultaneous serum phosphate. We used $FEPO_4$ instead of the renal threshold of phosphate reabsorption standardized to glomerular filtration rate (TmPi/glomerular filtration rate), as the latter has been validated for use only in fasting subjects[50] and our subjects were not asked to fast before sample acquisition. Osmotic diuresis after transplantation is a potential cause of hypophosphatemia that is independent of FGF-23 and PTH. Hypokalemia is a common consequence of osmotic diuresis. To assess whether hypophosphatemia represented nonspecific tubular wasting, we also examined potassium levels during the post-transplant period.

Statistical analysis

Patient characteristics are reported using standard descriptive statistics. Crude levels of analytes expressed as a function of time post-transplantation are presented in scatter plots. Pre-transplant values were considered to be collected at time = 0. Mixed model linear regression analyses that account for the repeated-measures

design were used to test our hypotheses that FGF-23 was more strongly associated with serum phosphate, $FEPO_4$, and calcitriol levels compared with PTH. In the models, time was treated as a linear covariate represented as the number of days post-transplant (random slopes models). Mixed-model regression analyses are robust to different numbers of follow-up observations within individual subjects.[51] Analyses that modeled calcitriol levels as the dependent variable were also adjusted for 25D levels. When necessary, squared and cubic terms were included in the regression models to account for exponential relationships between predictors and dependent variables.

To further assess the relative contributions of FGF-23 versus PTH in the development of post-transplant hypophosphatemia, we examined the risk of developing hypophosphatemia according to the pre-transplant FGF-23 and PTH levels, and according to the percent change from the pre- to the first post-transplant levels. We also examined the risk of developing hypophosphatemia according to the area under the FGF-23 and PTH curves spanning the early post-transplant period as a summary measure of overall initial exposure to these phosphaturic hormones. Analyses were performed using Stata Intercooled 7.0 (Stata Corporation, College Station, TX, USA). Two-sided *P*-values $\leqslant 0.05$ were considered statistically significant.

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